CD45 Molecule in γδ T-Cell Generation: Disruption of CD45 Exon 6 Does Not Affect Vγ3 Dendritic Epidermal T-Cell Development

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There are two distinct lineages of T cells: T-cell receptor (TCR) αβ-bearing cells (αβ T cells) and TCR γδ-bearing cells (γδ T cells). All of the αβ T cells and most subsets of γδ T cells develop in the thymus. It has been demonstrated that the protein tyrosine phosphatase CD45 plays a pivotal role in the intrathymic development of αβ T cells. Thymocyte maturation is arrested at the transitional stage from immature CD4+ CD8+ double-positive to mature CD4+ or CD8+ single-positive cells after CD45 exon 6 gene disruption. In this study, we examined whether Vγ3 dendritic epidermal T cells (DETC), a subset of thymus-dependent γδ T cells uniquely residing in the murine epidermis, are altered in the CD45 exon 6–deficient mice. *In situ* immunolabeling on epidermal sheets demonstrated that the CD45-deficient mice had a normal density and immunophenotype of Vγ3 DETC compared with the wild-type control mice. Reverse transcriptase polymerase chain reaction revealed that similar levels of Vγ3 TCR mRNA were present in the epidermis of CD45-deficient mice and wild-type controls. Flow cytometry demonstrated no significant difference in the proportion of Vγ3 T cells in the epidermis between the genotypes. In addition, Vγ2 T cells, another subset of γδ T cells, were also examined by flow cytometry. The frequency of Vγ2 T cells in lymph nodes was normal in the CD45-deficient mice. Our results indicate that although CD45 is crucial for the development of αβ T cells, this molecule is not necessary for the thymic maturation of γδ T cells, including Vγ3 DETC and Vγ2 T cells. ***Key words: tyrosine phosphatase/gene knockout/mice/Thy-1+ cell. J Invest Dermatol 108:49–52, 1997***

There are two distinct lineages of T cells: αβ T-cell receptor (TCR)-bearing cells (αβ T cells) and γδ TCR-bearing cells (γδ T cells). The overwhelming majority of dendritic epidermal T cells (DETC) in the murine skin belong to the γδ T-cell lineage and express TCR composed of Vγ3, Jγ1, Cγ1/Vδ1, Dδ2, Jδ2, Cδ chains (Asarnow *et al*, 1989). Although it is known that Vγ3 DETC precursor arises in the thymus, the developmental stages that Vγ3 DETC undergo remain unclear (Tigelaar and Lewis, 1995). The CD45 molecule is thought to be important for the development of αβ T cells, but the role of CD45 in γδ T-cell development is less clear.

CD45 is a transmembrane protein tyrosine phosphatase (PTPase) expressed on all nucleated hematopoietic cells and their precursors (Thomas, 1989). CD45 molecule plays a pivotal role in T-cell development (Trowbridge and Thomas, 1994). T-cell maturation in the thymus involves a selection mechanism whereby certain cells survive whereas others are destroyed. The selection processes preserve the developing T cells that express self-major histocompatibility complex–restricted, foreign antigen–specific TCRs (positive selection) and eliminate autoreactive cells (negative selection). Although it is well known that positive and negative selection processes are crucial in the shaping of the αβ T-cell repertoire, the role of selection in the generation of γδ T cells remains unclear. CD45 has been demonstrated to play an important role in the transitional stage of CD4+ CD8+ double-positive to CD4+ or CD8+ single-positive thymocytes in the maturation of αβ T cells (Kishihara *et al*, 1993). In this study, we investigated the role of CD45 in the development of γδ T cells using CD45-deficient mice. In particular, we examined Vγ3 DETC and TCR Vγ2+ cells in CD45-deficient mice by *in situ* immunophenotyping, flow cytometry, and reverse transcriptase polymerase chain reaction.

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Abbreviation: PTPase, protein tyrosine phosphatase.

MATERIALS AND METHODS

Mice CD45 gene-targeted mutant (knockout) mice (−/− mice) were kindly supplied by Dr. T.W. Mak (Amgen Institute, University of Toronto, Canada) and were maintained under a specific pathogen-free environment at the animal colony (Sunnybrook Health Science Center, University of Toronto). The knockout mice were generated by gene disruption of CD45 exon 6 and back-crossed more than five times to C57BL/6, as described previously (Kishihara *et al*, 1993). C57BL/6 mice were purchased from the Charles River Breeding Laboratories (Quebec, Canada) and were used as a wild-type control (+/+ mice). All mice were used at 8-12 wk of age.

Antibodies Monoclonal antibodies (MoAbs), including anti-Vγ3 TCR, -Vγ2 TCR, -γδ TCR, anti-Vγ3 TCR/fluorescein isothiocyanate (FITC), anti-αβ TCR/FITC, anti–Thy-1.2/phycoerythrin, and anti-CD32/CD16 (to block FcγR II/III), and isotype controls were purchased from Pharmingen (San Diego, CA). Anti-CD3, -CD4, -CD8, anti–Thy-1.2, and anti-CD3/PE were purchased from Serotec (Toronto, ON). Secondary antibodies, including goat anti-rat IgG/biotin, goat anti-hamster IgG/biotin, and

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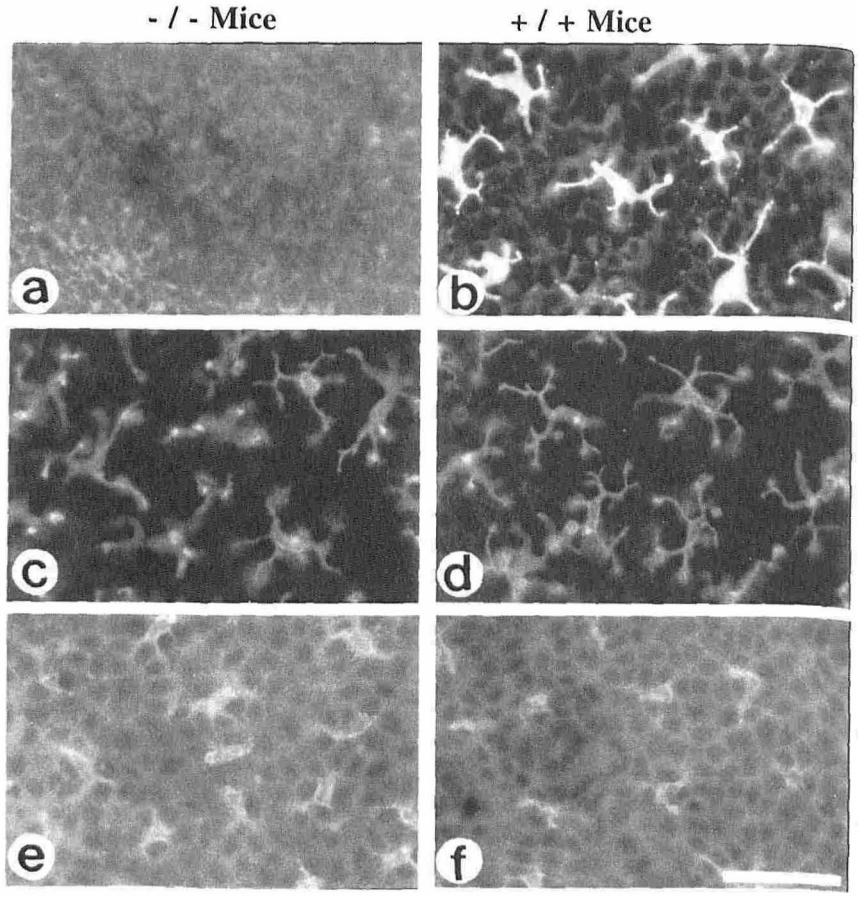

Figure 1. A normal number of Vγ3 TCR+ cells is present in the epidermis of CD45-deficient mice. Epidermal sheets obtained from CD45-deficient mice and the wild-type mice were immunolabeled with MoAbs against CD45 and Vγ3 TCR. CD45+ cells were absent in the epidermis of CD45 −/− mice *(a)*, whereas CD45+ cells were present in the wild-type controls *(b)*. A three-step labeling procedure with anti-Vγ3 TCR revealed no significant difference in the density of Vγ3+ cells between CD45 −/− mice *(c)* and +/+ mice *(d)* ($p > 0.05$). Although the staining was weaker by direct labeling, similar numbers of labeled cells were seen in both genotypes *(e,f)*. *Scale bar*, 50 μm.

streptavidin/FITC, were purchased from Cedarlane Laboratories (Hornby, ON). Goat anti-mouse IgM/biotin and ExtraAvidin/TRITC were purchased from Sigma (St. Louis, MO).

***In Situ* Immunolabeling** Epidermal sheets were obtained from the ears of mice by a separating technique using 0.5 M ammonium thiocyanate or 1% dispase (Matsue *et al*, 1992; Kilgus *et al*, 1993). Acetone-fixed epidermal sheets were labeled with a three-step immunolabeling procedure, as described previously (Wang *et al*, 1992). Briefly, epidermal sheets were first incubated with MoAbs overnight at room temperature, then washed with phosphate-buffered saline and reacted with a biotin-conjugated secondary antibody at 37°C for 1 h. After washing with phosphate-buffered saline, the epidermal sheets were incubated with streptavidin/FITC at 37°C for 1 h. Alternatively, epidermal sheets were directly labeled with anti-Vγ3 TCR/FITC overnight at room temperature. Positive labeled dendritic epidermal cells were counted in ten random fields with the aid of a micrometer grid. For double immunolabeling, epidermal sheets were incubated with MoAbs (e.g., anti-CD3) as described earlier, then reacted with biotin-conjugated secondary antibodies and visualized by ExtrAvidin/TRITC, and finally double labeled with anti-Vγ3 TCR/FITC.

Flow Cytometry Epidermal sheets obtained from the ears and trunk skin were treated with 0.1% trypsin and 0.025% DNase for 10 min at 37°C. Epidermal cell suspensions were prepared and incubated overnight in the presence of recombinant human interleukin-2 (20 U per ml) to allow the recovery of TCR expression (Correa *et al*, 1992). The cell suspensions were then subjected to a 15-min density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories). Lymph node cell suspensions were prepared from the inguinal and axillary lymph nodes, as described previously (Wang *et al*, 1996). Cells (10^6) were incubated for 5 min on ice with anti-CD32/CD16 to block FcγR II/III, then reacted with anti-Vγ3 TCR/FITC (for epidermal cells) or with anti-Vγ2 TCR/FITC (for lymph node cells) for 45 min on ice. After washing, the cells were reacted with anti-Thy-1.2/PE or anti-CD3/PE for another 45 min on ice. Two-color immunofluorescence was analyzed on a Coulter EPICS 75L flow cytometer (Hialeah, FL). A minimum of 20,000 cells were analyzed for each sample.

Reverse Transcriptase-Polymerase Chain Reaction Oligonucleotides were synthesized by Dalton Chemical Laboratories Inc. (North York, ON, Canada). Primers for TCR Vγ3 were: 5′-TGT GCA CGT GTA CCA ACT GA-3′ (sense) and 5′-CAG AGG GAA TTA CTA TGA GC-3′ (anti-sense) (Erb *et al*, 1995). Primers for β-actin were: 5′-GTG GGC CGC TCT AGG CAC CAA-3′ (sense) and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ (anti-sense). Total RNA was extracted from epidermal cell suspension, and from thymus and liver as controls (Chomczynski and Sacchi, 1987); cDNA synthesis and amplification were then performed (Kondo *et al*, 1993).

RESULTS

Density of Vγ3 TCR+ Cells Is Normal in the Epidermis of CD45-Deficient Mice The deficiency of CD45 molecules was confirmed by *in situ* immunolabeling with anti-CD45 MoAb on the epidermal sheets from CD45-deficient mice. CD45+ dendritic cells were absent in the epidermis of CD45 −/− mice **(Fig 1*a*)**, whereas numerous CD45+ dendritic cells, representing DETC and Langerhans cells, were present in the epidermis of wild-type mice **(Fig 1*b*)**. Epidermal sheets stained with anti-Vγ3 TCR by a three-step labeling procedure demonstrated a population of Vγ3 TCR+ cells with highly dendritic morphology present in both CD45 −/− mice **(Fig 1*c*)** and CD45 +/+ mice **(Fig 1*d*)**. There was no significant difference in the density of Vγ3 TCR+ cells between the CD45-deficient mice and the wild-type mice (417 ± 52 cells/mm^2 *vs* 423 ± 41 cells/mm^2, mean ± SD; $p > 0.05$).

Epidermal sheets stained directly with anti-Vγ3 TCR/FITC demonstrated Vγ3 TCR+ cells in both genotypes; however, labeling was weaker than the staining with the three-step procedure, and the cells appeared less dendritic. Nevertheless, the number of Vγ3 TCR+ cells present in the CD45-deficient mice **(Fig 1*e*)** was similar to that in the wild-type mice **(Fig 1*f*)**.

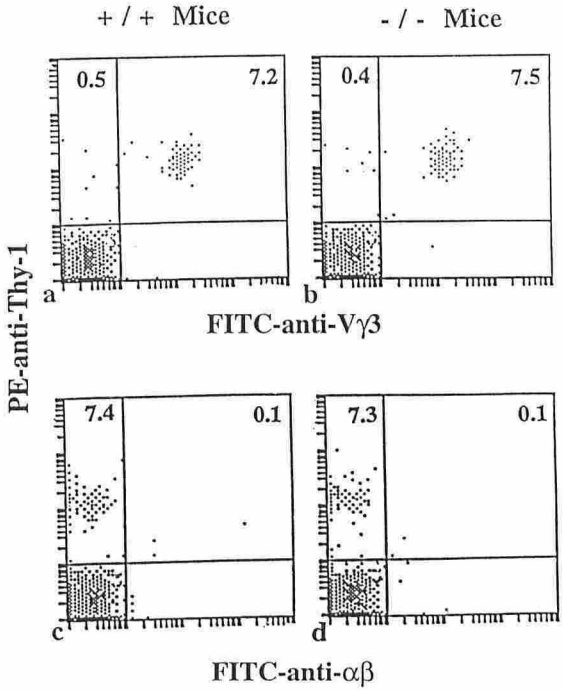

Figure 2. Flow cytometric analysis demonstrates a normal proportion of Vγ3 DETC in the epidermal cells of CD45-deficient mice. Epidermal cell suspensions obtained from CD45-deficient mice and the wild-type mice were double immunolabeled with anti–Thy-1.2/PE *versus* anti-Vγ3 TCR/FITC *(a,b)* or anti-αβ TCR/FITC (control) *(c,d)*, as described in *Materials and Methods*. Cell suspensions were analyzed by two-color flow cytometry. A minimum of 20,000 cells were analyzed for each sample on a Coulter EPICS 75L flow cytometer. A similar proportion of Thy-1+/Vγ3 TCR+ double-positive cells (DETC) existed in the wild-type mice *(a)* and in CD45-deficient mice *(b)*.

Phenotypic Characterization of Vγ3 TCR+ Cells Phenotypic analysis of Vγ3 TCR+ cells with double immunolabeling demonstrated that these cells were CD3+, CD4−, CD8−, Thy-1+, and γδ TCR+ in both CD45-deficient mice and wild-type mice, confirming that they were DETC. CD45 molecule was also expressed on Vγ3 TCR+ cells in the wild-type controls, but not in CD45-deficient mice.

Abundant TCR Vγ3 Transcripts Are Present in CD45-Deficient Mice To investigate whether the expression of TCR Vγ3 mRNA was normal in the CD45-deficient mice, we performed reverse transcriptase-polymerase chain reaction. Abundant TCR Vγ3 transcripts were detected in the epidermis from both CD45 −/− mice and +/+ mice, but not in the liver or thymus. No significant difference in the level of TCR Vγ3 mRNA was detected between the genotypes.

Proportion of Vγ3 DETC in Epidermal Cells Is Normal in CD45-Deficient Mice To determine the percentage of Vγ3 DETC among the epidermal cells, we detected Thy-1 and Vγ3 TCR double-positive cells by two-color flow cytometry. To remove the dead cells and debris, epidermal cell suspensions were gradient centrifuged on lympholyte-M before immunolabeling. As shown in **Fig 2**, epidermal cell suspensions from CD45-deficient mice contained normal proportions of Vγ3 TCR+/Thy-1+ double-positive cells (DETC) as compared with the wild-type mice.

Vγ2 T Cells Develop Normally in CD45-Deficient Mice To examine whether CD45 molecules affect other subsets of γδ T cells, we detected Vγ2 T cells by two-color flow cytometry in the lymph nodes from CD45-deficient mice. About 1.2-2.3% of lymph node cells were Vγ2 TCR+/CD3+ double-positive cells in both CD45 −/− mice and the +/+ mice. No significant difference in the frequency of Vγ2 T cells was detected between these genotypes.

DISCUSSION

In 1983, investigators demonstrated that in addition to Langerhans cells, the murine epidermis harbors a population of Thy-1+, major histocompatibility complex class II− dendritic cells (Bergstresser *et al*, 1983; Tschachler *et al*, 1983). These cells were initially termed Thy-1+ dendritic epidermal cells (Thy-1+ DEC). With the demonstration that Thy-1+ DEC express TCR, these cells have been identified as a subset of T-cell lineage and renamed dendritic epidermal T cells (DETC) (Stingl *et al*, 1987; Steiner *et al*, 1988). Further studies demonstrated that the majority of DETC bear an identical TCR, composed of Vγ3, Jγ1, Cγ1/Vδ1, Dδ2, Jδ2, Cδ gene segments (Asarnow *et al*, 1989). The exact function of these Vγ3 DETC remains unclear.

Vγ3 DETC develop in the fetal thymus. Early fetal thymocytes express the same TCR Vγ3, Jγ1, Cγ1/Vδ1, Dδ2, Jδ2, Cδ as DETC. Furthermore, the disappearance of Vγ3 TCR+ thymocytes directly precedes the first appearance of Vγ3 DETC in the epidermis, suggesting that they represent the precursor cells of DETC (Havran and Allison, 1988). Further evidence has been obtained from the observation that Vγ3 DETC are absent in the epidermis of athymic nu/nu mice. Transplantation of fetal thymus or injection of fetal thymocytes into young nu/nu mice or congenic adult mice results in the appearance of donor-type DETC in the epidermis of recipients (Havran and Allison, 1990; Payer *et al*, 1991).

Research shows that αβ T cells and γδ T cells are products of separate lineages that arise from common progenitors; the lineages segregate before the onset of TCR gene rearrangements (Hass and Tonegawa, 1992). The αβ cell lineage and γδ cell lineage may diverge at a stage just before the expression of CD4 and CD8 in the αβ cell lineage (Petrie *et al*, 1992). The ontogenesis of αβ T cells is clear: αβ T cells develop from double-negative CD4− CD8− TCR− immature cells in the thymus, then progress to a double-positive CD4+ CD8+ TCR^{low} stage. Finally they undergo a selection process, and become mature single-positive CD4+ TCR^{high} or CD8+ TCR^{high} T cells (Shortman, 1992). The ontogenesis of γδ T cells, however, remains unclear. Although a small number of γδ TCR+ thymocytes express CD8 and even low levels of CD4, the development of γδ cells does not involve a CD4+ CD8+ double-positive stage (Itohara *et al*, 1989; Fisher and Ceredig, 1991).

CD45 is one of the most abundant leukocyte cell surface glycoproteins. CD45 is a PTPase and plays important roles in lymphocyte activation and thymic maturation. Antigen receptor-induced signal transduction in both T-cell and B-cell lines is crucially dependent on the transmembrane PTPase. Modulation of CD45 isoform expression may be involved in thymic selection events (Wallace *et al*, 1992). To investigate the *in vivo* role of CD45 in the development of lymphocytes, Kishihara *et al* (1993) generated CD45-deficient mice by gene disruption of CD45 exon 6. These mutant mice lacked CD45 expression on B cells and on the majority of thymocytes and peripheral T cells. In these mice, B lymphocytes develop normally, but T-lymphocyte development is impaired. Thymocyte maturation is blocked at the transitional stage from immature double-positive CD4+ CD8+ to mature single-positive CD4+ or CD8+ cells, and thus the number of peripheral T cells is dramatically decreased. These observations suggest that CD45 PTPase plays a crucial role in the development of αβ T cells.

Whether CD45 PTPase also plays a role in the development of γδ T cells remains unclear. Recently, Kawai *et al* (1995) reported that the development of Vγ3 DETC was impaired in CD45 PTPase-deficient (exon 6 gene-targeted) mice as well as in $p56^{lck}$ protein tyrosine kinase–deficient mice. Both protein tyrosine kinases and PTPases play important roles in signal transduction during thymocyte development, but they act at different times. Unlike the late block in thymocyte development in CD45-deficient mice, the block in $p56^{lck}$-deficient mice occurs at an early stage (before positive and negative selection processes begin) (Molina *et al*,

1992). Further studies demonstrate that the thymic development of transgenic γδ T cells is greatly impaired in $p56^{lck}$-deficient mice (Penninger *et al*, 1993). Therefore, it is not surprising that the maturation of both αβ cells and γδ cells is impaired after $p56^{lck}$ gene disruption. We were surprised, however, by the finding that CD45 affected the development of γδ T cells.

In contrast to the finding of Kawai *et al* (1995), our study demonstrated no significant differences in the density, morphology, or phenotype of Vγ3 DETC between CD45 −/− mice and wild-type mice. The discrepancy between the groups may be related to the experimental protocol. In our study for direct immunostaining, we incubated the epidermal sheets with FITC-conjugated anti-Vγ3 TCR MoAb overnight at room temperature, whereas Kawai *et al* incubated the sheets for only 1 h at 37°C. The staining of Vγ3 DETC was weaker and the dendritic processes were less obvious with the direct immunolabeling technique than with indirect immunolabeling. Nevertheless, a similar number of Vγ3+ dendritic cells were found in the epidermis in CD45-deficient mice and in the wild-type mice. When the epidermal sheets were immunolabeled by a three-step procedure, the Vγ3 DETC were labeled more intensely and the dendrites were more apparent, as expected. To examine further whether these anti-Vγ3 TCR-reactive cells were DETC, we performed double labeling. Phenotypically, all of the Vγ3 TCR+ cells were CD3+, CD4−, CD8−, γδ TCR+, and Thy-1+, confirming that they were DETC. To evaluate the proportion of Vγ3 DETC in the total epidermal cells, we performed flow cytometric analysis on epidermal cell suspension. The result revealed that the percentage of Vγ3 DETC among epidermal cells was similar in CD45-deficient mice and the wild-type mice.

To investigate whether CD45 deficiency affects the maturation of other subsets of γδ T cells besides Vγ3 DETC, we examined Vγ2 T cells by flow cytometry. Vγ2 T cells represent another subset of γδ T cells located mainly in the secondary lymphoid organs. We found that there was no significant difference in the proportion of Vγ2 T cells in the lymph node cells between CD45 −/− mice and the +/+ mice.

Taken together, our observations demonstrate that the disruption of CD45 exon 6 does not block the development of Vγ3 DETC or Vγ2 T cells, suggesting that CD45 PTPase is not necessary for the development of γδ T cells.

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